

Research Article

Curcumin Attenuates Gastric Cancer Induced by *N*-Methyl-*N*-Nitrosourea and Saturated Sodium Chloride in Rats

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To determine effects of curcumin on *N*-methyl-*N*-nitrosourea (MNU) and saturated sodium chloride (s-NaCl)-induced gastric cancer in rats. Male Wistar rats were divided into 5 groups: control (CO), control supplemented with 200 mg/kg curcumin (CC), MNU + s-NaCl, MNU + s-NaCl supplemented with 200 mg/kg curcumin daily for the first 3 weeks (MNU + s-NaCl + C3W), and MNU + s-NaCl supplemented with curcumin for 20 weeks (MNU + s-NaCl + C20W). To induce stomach cancer, rats except for CO and CC were orally treated with 100 mg/kg MNU on day 0 and 14, and s-NaCl twice-a-week for the first 3 weeks. The experiment was finished and rats were sacrificed at the end of 20 weeks. Cancers were found in forestomachs of all rats in MNU + s-NaCl. The expressions of phosphorylated inhibitor kappaB alpha (phospho-I κ B α), 8-hydroxy-2'-deoxyguanosine (8-OHdG), and cyclin D1 significantly increased in MNU + s-NaCl compared with CO. Curcumin treatments for 3 and 20 weeks reduced the cancer incidence resulting in a decrease of phospho-I κ B α expression in benign tumor-bearing rats compared with MNU + s-NaCl. Curcumin treatment for 20 weeks also decreased 8-OHdG expression in benign tumor-bearing rats compared with MNU + s-NaCl. Curcumin can attenuate cancer via a reduction of phospho-I κ B α and 8-OHdG expressions, which may play a promising role in gastric carcinogenesis.

1. Introduction

Gastric cancer can generate in any part of the stomach. Poorly detected, gastric cancer causes nearly one million annual deaths worldwide [1, 2]. Gastric cancer is closely associated with dietary factors and *Helicobacter pylori* infection. Previous studies have reported that consumption of salty foods and *N*-nitroso compounds and a low intake of fresh fruits and vegetables increases the risk of gastric cancer [3, 4]. Hypertonic NaCl solutions induce gastric cancer in animal models through the enhancement of tissue damage resulting in cell proliferation [5]. Experimental animal models have also provided support for the hypothesis that salt promotes gastric carcinogenesis induced by *N*-nitroso carcinogen, such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) or *N*-methyl-*N*-nitrosourea (MNU) [6–9]. MNU, a mutagen and genotoxic substance, is a potent inducer of cellular stress leading to chromosomal aberrations, point mutations, cell death, and DNA damage [10]. Other studies reported that

administration of MNU by oral gavage induced gastric cancer in rats and mice [11, 12]. Thus, the association between saturated NaCl and *N*-nitroso carcinogen could promote gastric carcinogenesis in rats by inflammation, mutation, and compensatory cell proliferation.

A range of stimuli such as reactive oxygen species (ROS) and cytokines from inflammatory response can activate inhibitor kappaB kinase (IKK) complex resulting in inhibitor kappaB (I κ B) phosphorylation and proteolysis. Phosphorylation of I κ B α elicits I κ B α degradation, allowing the nuclear translocation of nuclear factor kappaB (NF- κ B) complex and activation of target genes that are involved in the control of cellular proliferation and carcinogenesis such as cyclin D1 [13, 14]. The interaction of ROS with the nucleobases of the DNA strand, such as guanine, leads to the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and structural alteration in DNA. The 8-OHdG is a potential biomarker of oxidative damage of DNA and a factor of initiation and promotion of carcinogenesis [15]. These molecules play

a pivotal role in carcinogenesis and may be targets for therapeutic approaches.

Chemoprevention is promising as a preventive approach for cancers. Curcumin (diferuloylmethane), a polyphenol compound, is an active ingredient of tumeric (*Curcuma longa*). Curcumin has chemopreventive properties. Importantly, curcumin is safe for humans and animals [16]. Curcumin shows beneficial effects in many cancers including colorectal cancer, breast cancer, skin cancer, and oral cancer [17]. Several signalling pathways implicated in carcinogenesis including NF- κ B signalling have been modulated by curcumin treatment [18]. However, data concerning the effect of curcumin on *in vivo* study of gastric cancer and key proteins involved in carcinogenesis induced by MNU and saturated NaCl (s-NaCl) have not been confirmed.

Therefore, the present study aims to examine the protective effect of curcumin on gastric cancer in rats induced by MNU and s-NaCl administration. In addition, activation of NF- κ B, expressions of oxidative damage of DNA and cell cycle regulator cyclin D1 will be investigated.

2. Methods

2.1. Experimental Design. 6-week-old male Wistar rats (National Laboratory Animal Centre, Mahidol University, Bangkok, Thailand) were used. All experiments and procedures carried out on the animals were approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Rats were housed in a controlled temperature room at $25 \pm 1^\circ\text{C}$ under standard conditions (12-h dark-light cycle). Thirty rats were randomized into five groups (six rats each) as follows.

Control rats (CO) were fed citrate buffer, pH 4.5 (1 mL/rat) orally via intragastric tube on days 0 and 14 of the experiment. In addition, rats were fed normal saline (1 mL/rat) orally twice a week for the first 3 weeks of the experiment. Corn oil (2.5 mL/kg) was administered daily by intragastric tube for 20 weeks.

Control rats supplemented with curcumin (CC) were fed citrate buffer, pH 4.5 and normal saline as previously described. 200 mg/kg curcumin (95% purified curcumin, Cayman Chemical, MI, USA) was dissolved in corn oil and given daily to rats by intragastric tube for 20 weeks.

MNU- and saturated NaCl-induced rats (MNU + s-NaCl) were treated for gastric carcinogenesis by MNU (Sigma-Aldrich, MO, USA) and s-NaCl (Merck, Germany) according to Thong-Ngam et al. [19]. Briefly, rats were fed 100 mg/kg MNU (dissolved in citrate buffer, pH 4.5) via intragastric tube on days 0 and 14 of the experiment. In addition, rats were fed s-NaCl (30% NaCl solution, 1 mL/rat) orally twice weekly for the first 3 weeks of the experiment. Corn oil was administered daily by intragastric tube for 20 weeks.

MNU- and s-NaCl-induced rats, supplemented with curcumin for 3 weeks (MNU + s-NaCl + C3W), were given with MNU and s-NaCl as previously describe. The 200 mg/kg curcumin was fed daily to rats by intragastric tube during administration of MNU and s-NaCl for the first 3 weeks.

MNU- and s-NaCl-induced rats, supplemented with curcumin for 20 weeks (MNU + s-NaCl+C20W), were induced with MNU and s-NaCl. The 200 mg/kg curcumin was fed daily to rats by intragastric tube for 20 weeks.

2.2. Stomach Tissues Preparation. After 20 weeks, all animals were sacrificed by intraperitoneal injection of Thiopental (Abbott, Italy, 120 mg/kg) after overnight fasting. Then, the stomach was excised and divided into 2 parts symmetrically along the greater and lesser curves. One part was fixed in liquid nitrogen and kept at -80°C for western blot analysis. Another part was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for histological study.

2.3. Histopathological Study. The tissue was fixed with 4% paraformaldehyde fixed and paraffin embedded. Multiple 2 μm -thick histological sections were stained with hematoxylin and eosin (H&E). The alterations of gastric epithelial cells and the incidence of gastric carcinogenesis were determined by a pathologist. In addition, the cancer incidence was calculated as percentage using the following formula:

$$\text{Cancer incidence} = 100 \times \left(\frac{\text{numbers of cancer-bearing rats}}{\text{numbers of induced rats}} \right). \quad (1)$$

2.4. Western Blot Analysis. The tissue sample (0.05 g) was homogenized in 0.5 mL of ice-cold lysis RIPA buffer (Cell Signalling Technology Inc., MA, USA) with protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Sigma-Aldrich). The homogenate was sonicated for 15 seconds and centrifuged at 11,000 g for 10 minutes at 4°C . The supernatant was retained. Protein concentration was measured with the BCA Protein Assay Kit (Thermo Scientific). The extracted proteins were mixed with loading buffer and boiled for 5 min. Then, the extracted proteins (35 μg /lane) were applied to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membrane (Pall Corporation, FL, USA). The blotted membrane was incubated with 5% nonfat dried milk in TBS (0.02 M Tris, pH 7.6, and 0.15 M NaCl) for 1 hour at room temperature. Then, the membrane was probed with mouse monoclonal anti-phosphorylation of inhibitor kappaB alpha (Phospho-I κ B α , Ser32/36) antibody (1 : 1000; Cell Signalling Technology Inc.) overnight at 4°C . Moreover, the membrane was also probed with anti- β -actin antibody (sc-47778, 1 : 5,000; Santa Cruz Biotechnology Inc., CA, USA) for 1 hour at room temperature. After three-time washing in TBS/0.01% Tween-20, the membrane was incubated with goat anti-mouse IgG HRP secondary antibody (1 : 4,000; Cayman Chemical) for 1 hour at room temperature. The bands of protein expression were developed with a commercial chemiluminescence detection kit (Amersham ECL plus western blotting system, GE Healthcare, UK). The luminescence was exposed to film (Fujifilm, Japan). Expression levels of proteins were quantified by ImageJ program (US National Institutes of Health, Bethesda, MA, USA).

TABLE 1: Histopathological changes of gastric mucosa in the experimental groups.

Group	Normal	Mucosal congestion	Benign papillary growth	Squamous cell carcinoma			Cancer incidence (%)
				Submucosal invasion	Muscle invasion	Serosal invasion	
CO (N = 6)	6	—	—	—	—	—	0
CC (N = 6)	6	—	—	—	—	—	0
MNU + s-NaCl (N = 6)	—	—	—	2	3	1	100
MNU + s-NaCl + C3W (N = 5)	—	—	2	2	1	—	60
MNU + s-NaCl + C20W (N = 6)	—	1	2	3	—	—	50

The level of phospho-I κ B α (Ser32/36) expression was normalized by β -actin density.

2.5. Immunohistochemistry. In this study, we used BenchMark XT Instrument (Ventana, Medical System Inc., AZ, USA). Immunostaining for 8-OHdG or cyclin D1 was performed in paraffin embedded sections by the following processes. Briefly, the tissue sections were deparaffinized with EZ prepTM. After that, the sections were retrieved the antigen (8-OHdG or cyclin D1) with Sodium Chloride Sodium Citrate pH 6.5–7.5 (SSCTM). Next, 1% Hydrogen peroxide (H₂O₂, UltraViewTM Inhibitor) was used to block endogenous peroxidase activity. Then, the primary antibody used for 8-OHdG (1 : 400; Japan Institute for the Control of Aging, Japan) or cyclin D1 (1 : 200; Thermo Scientific, MI, USA) was applied and incubated at 37°C for 60 minutes or 32 minutes, respectively. After that, the goat anti-Mouse IgG (UltraViewTM HRP Multimer) was used as secondary antibody. Color was developed by UltraViewTM DAB chromogen, UltraViewTM H₂O₂, and UltraViewTM copper. Then, the slides were counterstained with Hematoxylin II and Lithium Carbonate. Under light microscope (Nikon E50i, Nikon Corporation, Japan), immunoreactive cells of 8-OHdG and cyclin D1 were defined as those with dark-brown-stained nuclei of gastric epithelial cells. To verify the expressions of 8-OHdG and cyclin D1 in all animals, digital images were taken in high magnification field (400x) from each sample using a microscope equipped with digital camera (Nikon Digital Sight DS-Fi1, Nikon Corporation, Japan). Ten images from two sections per animals were analyzed. The numbers of dark brown stained in nuclei of epithelial cells were counted manually using Point tool in the IMAGE-PRO PLUS software program (version 6.1). A thousand gastric epithelial cells were counted for each rat. The data were shown as the percentage (%) of immunoreactive cells calculating from following equation:

$$\begin{aligned} &\text{The percentage of immunoreactive cells (\%)} \\ &= \left(\frac{\text{number of nuclei stained cells}}{\text{number of examined cells}} \right) \times 100\%. \end{aligned} \quad (2)$$

2.6. Statistical Analysis. Statistical analyses were conducted using Fisher's exact test for incidence of gastric cancer. Phospho-I κ B α , 8-OHdG, and cyclin D1 results were shown as mean \pm SD and analyzed with one-way ANOVA and LSD

post hoc test. For all comparisons, a *P* value of less than 0.05 was considered to be statistically significant. All the statistical tests were performed using the computer program SPSS, version 13.0, for Windows (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Gastric Cancer Incidence and Histopathological Studies of MNU and s-NaCl-Associated Gastric Carcinogenesis and Effects of Curcumin. During the whole-study period, there was only one rat death. At 3 weeks, the rat in MNU + s-NaCl + C3W died from aspiration after feeding. The results of autopsy showed congestion and bleeding spots in both lungs. There was hyperkeratosis in forestomach mucosa, but no precancerous lesions or tumor was detected by histological examination. 29 rats survived the 20 weeks of the study.

Gastric cancer incidence was shown in Table 1. There was no squamous cell carcinoma (SCC) in CO and CC groups. In MNU + s-NaCl group, SCC was found in forestomach of all rats (100%). SCC showed tumors scattered in the forestomach. In gross view, the tumor masses were coliform-like (Figure 1(a)). Histopathology of SCC showed dyskeratosis and invasion of cancerous tissue through all layers of stomach wall. The cancerous tissue invaded the submucosal layer (Figure 1(b)), the muscle layer (Figure 1(c)), or the serosa (Figure 1(d)). 3/5 (60%) rats in MNU + s-NaCl + C3W developed SCC. 2 rats in this group showed tumor-like lesions. Papillary growth or tumor-like lesion displayed hyperproliferation of epithelial cells, hyperkeratosis, but no invasion. These lesions were not diagnosed as cancers. In MNU + s-NaCl + C20W group, three rats developed SCC with submucosal invasion. The cancer incidence was 50%. One rat did not exhibit a cancerous lesion. Another 2 rats developed papillary growths.

3.2. Curcumin Supplementation for 3 and 20 Weeks Attenuated Development of Carcinogenesis Associated with Phospho-I κ B α Expression. From histopathological results (Table 1), rats in MNU + s-NaCl + C3W and MNU + s-NaCl + C20W groups were divided into subgroup C3W benign group (*N* = 2), C3W cancer group (*N* = 3), C20W benign group (*N* = 3), and C20W cancer group (*N* = 3). Results showed that the expression of phosphorylated I κ B α was not significantly different between CO and CC. Compared with CO, phosphorylated I κ B α expression in MNU + s-NaCl increased

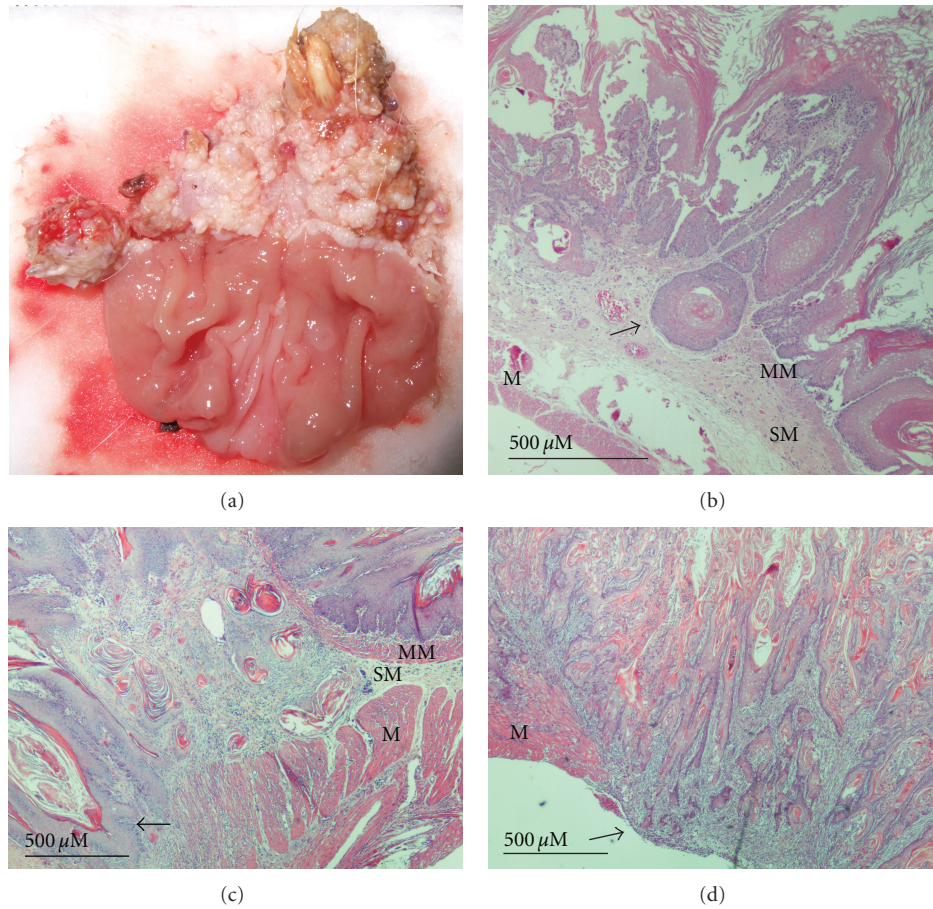


FIGURE 1: Macroscopic (a) and microscopic (b–d, H&E staining, 40x) appearance of squamous cell carcinoma (SCC) in the forestomachs. The multiple polypoid tumors developed in the stomach after MNU and saturated NaCl administration (a). SCCs in the gastric epithelium show the invasion of cancerous tissue with dyskeratosis through submucosal layer (b), muscle layer (c), or serosa (d). Note: the arrows indicate SCC with submucosal invasion (b), muscle-layer invasion (c), and stomach perforation (d). MM: muscularis mucosae, SM: submucosa, and M: muscle layer.

TABLE 2: The results of phospho-I κ B α -relative expression, 8-OHdG-immunoreactive cells (%), and cyclin D1-immunoreactive cells (%).

Parameters/Group	CO (N = 6)	CC (N = 6)	MNU + s-NaCl (N = 6)	MNU + s-NaCl + C3W (N = 5)		MNU + s-NaCl + C20W (N = 6)	
				C3W benign (N = 2)	C3W cancer (N = 3)	C20W benign (N = 3)	C20W cancer (N = 3)
Phospho-I κ B α	0.51 \pm 0.11 [†]	0.54 \pm 0.18 [†]	0.82 \pm 0.18*	0.46 \pm 0.04 [†]	0.60 \pm 0.29	0.51 \pm 0.08 [†]	1.16 \pm 0.34 ^{**,†}
8-OHdG	44.42 \pm 3.41 ^{††}	40.39 \pm 3.53 ^{††}	53.06 \pm 5.96 ^{**}	49.28 \pm 7.43	48.06 \pm 3.47	44.99 \pm 3.51 [†]	47.77 \pm 2.55
Cyclin D1	38.58 \pm 5.37 ^{††}	42.53 \pm 6.90 ^{††}	54.91 \pm 7.93 ^{**}	4.33 \pm 1.27*	54.92 \pm 3.87 ^{**}	45.31 \pm 6.29	53.82 \pm 11.01 ^{**}

These data were presented as the mean \pm SD. *Represented significant difference compared with CO group ($P < 0.05$). **Represented significant difference compared with CO group ($P < 0.01$). [†]Represented significant difference compared with MNU + s-NaCl group ($P < 0.05$). ^{††}Represented significant difference compared with MNU + s-NaCl group ($P < 0.01$).

significantly ($P = 0.010$). Curcumin supplementations for 3 and 20 weeks in C3W benign and C20W benign groups significantly declined the expression of phosphorylated I κ B α compared with MNU + s-NaCl ($P = 0.028$ and $P = 0.031$, resp.) (Table 2). The represented bands of phospho-I κ B α and β -actin were shown in Figure 2.

3.3. Curcumin Supplementation for 20 Weeks Attenuated Development of Carcinogenesis Associated with 8-OHdG and Cyclin D1 Expressions. 8-OHdG and cyclin D1 expressions

were studied by immunohistochemistry and shown as nuclei-stained cells. The average percentages of immunoreactive cells of all groups were shown in Table 2. From the results, the mean percentages of 8-OHdG and cyclin D1-immunoreactive cells were not significantly different between CO and CC. They were significantly increased in MNU + s-NaCl compared with CO ($P = 0.002$ and $P = 0.000$, resp.). Curcumin supplementation for 20 weeks reduced gastric cancer incidence and significantly decreased 8-OHdG expression in C20W benign group compared with

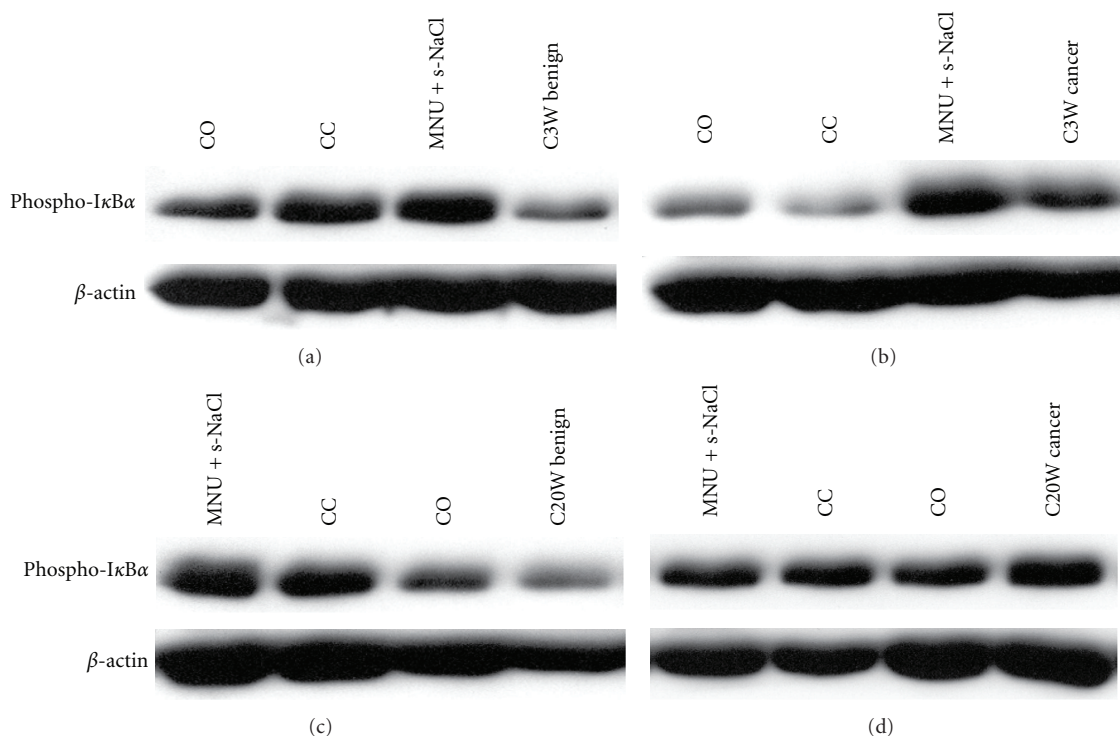


FIGURE 2: Western blot analysis of phospho-I κ B α expression. Curcumin supplementation in C3W benign group declined the expression of phosphorylated I κ B α compared with MNU + s-NaCl (a), whereas the expression of phosphorylated I κ B α in C3W cancer group did not decrease (b). Curcumin supplementation for 20 weeks in C20W benign decreased the expression of phosphorylated I κ B α compared with MNU + s-NaCl (c), whereas the expression of phosphorylated I κ B α in C20W cancer group did not decline (d). An antibody for β -actin was used as an internal control.

MNU + s-NaCl ($P = 0.015$) (Table 2). The average percentage of cyclin D1 stained cells in the C20W benign group tended to reduce, but not reach a statistically significant level when compared with MNU + s-NaCl ($P = 0.062$; Table 2). 8-OHdG was expressed primarily in gastric epithelial cells (Figure 2(a)). This expression was increased in the mucosa of SCC in both MNU + s-NaCl and C20W cancer groups (Figures 3(b) and 3(c)). This enhanced expression was limited to a small number of epithelial cells in C20W benign group (Figure 3(d)).

4. Discussion

This study demonstrated that oral gavage of MNU- and s-NaCl-induced a 100% cancer incidence in rats. The histological results showed that curcumin could attenuate the gastric carcinogenesis induced by MNU and s-NaCl in rats. Administration of curcumin in both MNU + s-NaCl + C3W and MNU + s-NaCl + C20W groups showed 40% and 50% reduction of cancer incidence, respectively. These observations indicated that early administration of curcumin (during the first 3 weeks of cancer induction) might prevent the initiation of carcinogenesis. This is in agreement with previous studies. Feeding 0.5 and 2.0% of commercial grade curcumin in the diet during the initiation period (2 weeks before, during, and 1 week after benzo(a)pyrene administration) reduced the number of mice with forestomach tumors [20]. Daily feeding of 1 and 2 g/mL radix curcumae extract

solution during MNNG administration for 40 weeks also showed the reduction of tumor incidences in 10% NaCl and MNNG-induced gastric cancer in rats [21].

Phosphorylation of I κ B α could imply the activation of NF- κ B, which plays a major role in carcinogenesis [13, 14]. In gastric carcinoma patients, NF- κ B activation correlated with I κ B α phosphorylation and degradation [22, 23]. Our study showed that overexpression of phosphorylated I κ B α was associated with cancer. This finding coincided with other reports [24, 25]. Activation of NF- κ B appeared to be a key step of keratinocyte transformation into SCC in mice [24]. 49% of prostate adenocarcinoma patients showed NF- κ B overexpression that correlated with advanced tumor stage [25]. Curcumin has a chemopreventive property resulting in suppressing NF- κ B activation. Many studies confirmed that a pivotal role of curcumin is inhibiting IKK activity with declining I κ B α phosphorylation [26, 27]. Our results showed that curcumin supplementations for 3 and 20 weeks significantly decreased phosphorylated I κ B α in benign tumor-bearing rats. Curcumin supplementation in this study prevented carcinogenesis by declining I κ B α phosphorylation.

8-OHdG is a potential biomarker of oxidative DNA damage and a factor of initiation and promotion of carcinogenesis [15]. In this study, we also showed that 8-OHdG expression significantly increased in the MNU + s-NaCl group compared with CO group. This observation confirmed many previous results obtained from various types of cancers in patients. The level of 8-OHdG expression is

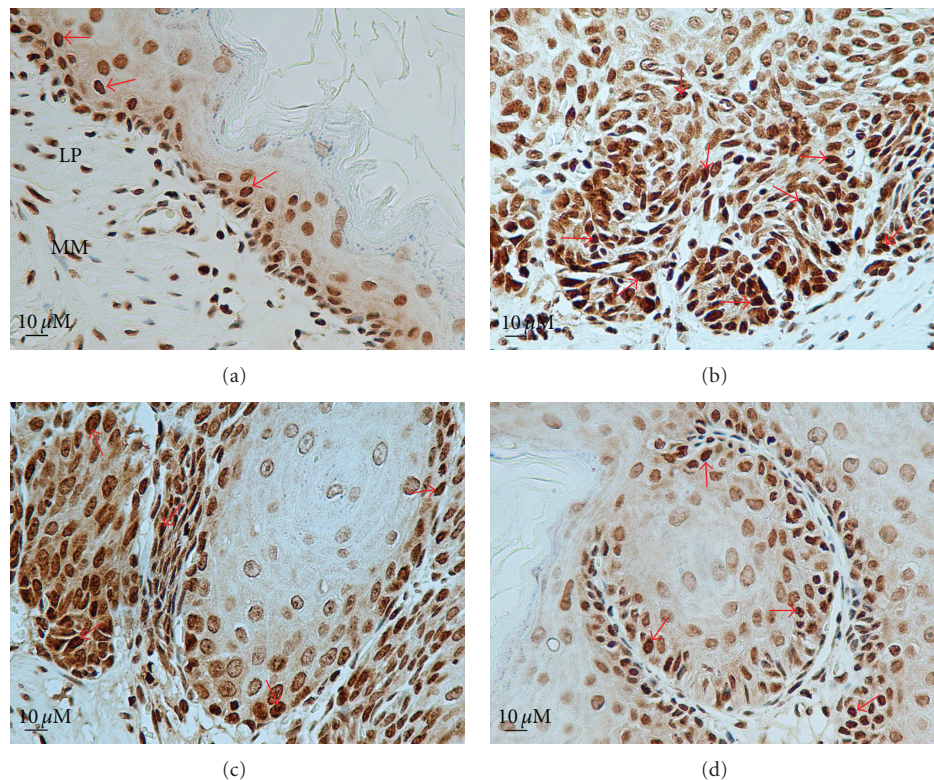


FIGURE 3: Immunohistochemical staining of 8-OHdG antibodies in the representative tissue specimens (400x): CO group (a), MNU + s-NaCl group (b), C20W cancer group (c), and C20W benign group (d). DAB stained immunoreactive cells (dark brown, arrows); nuclear counterstaining was performed with Hematoxylin II and Bluing reagent. LP: lamina propria and MM: muscularis mucosae.

elevated in colorectal cancer [28], hepatocellular carcinoma [29], oral SCC [30], and gastric cancer patients [31]. Considering the number of reports demonstrating a close relation between 8-OHdG formation and carcinogenicity, including this study, it is likely that 8-OHdG formations might participate in carcinogen-induced forestomach SCC. Curcumin showed a potent scavenger of reactive species (RS), such as superoxide anion, hydroxyl radical, singlet oxygen, nitric oxide, and peroxynitrite [32]. The reduction of RS could prevent the formation of 8-OHdG. This study demonstrated that 200 mg/kg curcumin supplementations for 3 and 20 weeks in MNU + s-NaCl-induced SCCs diminished 8-OHdG expression. In addition, 8-OHdG expression in C20W benign group was significantly reduced compared to MNU + s-NaCl. These results suggested that curcumin administration could protect carcinogenesis against formation of 8-OHdG.

Oxidative damage of DNA as well as deregulation of cell cycle control causes cancer. This study demonstrated that the immunoreactive cells of cyclin D1, the positive cell cycle regulator, significantly increased in MNU + s-NaCl compared with CO. The nuclear accumulation of cyclin D1 is an essential indicator of oncogenicity. Previous studies showed that nuclear immunoreactivity for cyclin D1 positively correlated with tumor cell proliferation of gastric cancer patients [33]. Moreover, cyclin D1 overexpression occurred in patients with oral SCC [34]. Cyclin D1 expression in the

C20W benign subgroup tended to decrease, but not reach a statistically significant level when compared with MNU + s-NaCl. This study suggested that curcumin could not prevent carcinogenesis through improvement of dysregulation of cell cycle as shown by cyclin D1 accumulation.

5. Conclusion

MNU and s-NaCl led to increase of phosphorylated I κ B α , 8-OHdG, and cyclin D1 that are associated with forestomach carcinogenesis. Concomitant treatment with 200 mg/kg curcumin during the first three weeks or the entire study period (20 weeks) could decrease cancer incidence to 40% and 50%, respectively. This treatment shows a significant reduction of phosphorylated I κ B α in benign rats. In addition, curcumin treatment for 20 weeks significantly reduces 8-OHdG expression. Hence, administration of curcumin during the initiation period could attenuate the incidence of cancer via reduction of phospho-I κ B α and 8-OHdG expressions.

Acknowledgments

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